Melamine, from fertilizer to pasture to cow's milk

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2010

Abstract

Title	:	Melamine, from fertilizer to pasture to cow's milk
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The aim of this study was to determine the rate of transfer of melamine as fertilizer ingredient to kikuyu pastures and if melamine would be transferred from the fertilized pasture to cow's milk. Three trials were conducted in the study, *viz.* a pilot pot plant trial, an applied pasture trial and a milk production trial.

Melamine is a commercially available industrial chemical with a high nitrogen content. Large quantities of melamine waste can sometimes be incorporated into crop and pasture fertilizers due to the high N content. An initial pot plant trial with kikuyu was conducted to determine whether melamine would be absorbed as such from the soil to the plant material. The pots were fertilized in the form of melamine adulterated Chinese maize Gluten 60, at a rate equivalent to 8.8 kg of melamine/ha. Results indicated that melamine was indeed absorbed and 7 days after fertilization, the concentration of melamine in the grass was 228 mg/kg.

An applied pasture trial was then conducted were three pastures of 0.3 ha each were used. One pasture served as a control and received N fertilization in the form of LAN at a rate of 40 kg N/ha. The other two pastures also received LAN, but with 10% (Treatment 1) and 20% (Treatment 2) of the LAN-N substituted with melamine-N. All three pastures also received P-fertilization in the form of Single Superphosphate at a rate of 20 kg P/ha and KCl fertilizer at a rate of 50 kg K/ha. Pasture samples were taken once a week for 10 weeks, each time at the exact same spot in each camp. Samples were dried and finely milled before analysis via LC-MS/MS for melamine content. The initial concentration of melamine in the grass of Treatment 2 was higher than that in the grass of Treatment 1. The rate at which melamine decayed in the plant material was found to be quite similar for the two melamine treatments. In this trial, melamine took around 10 weeks to reach undetectable levels in the grass. It was concluded that melamine was absorbed as such from the soil by pasture grass when included in a fertilizer.

For the milk production study, eighteen lactating Holstein cows, 60 ± 5.1 (SE) DIM, with a daily milk production of 36.5 ± 2.0 (SE) kg/d and weighing 609 ± 12.8 (SE) kg, were stratified according to

milk production and then randomly allocated to three groups of six cows. The groups were then randomly allocated to the three pastures used in the applied pasture trial. Cows were kept on the melamine fertilized pasture for 9 days, in which they were allowed to graze the pasture for approximately 10 hours each day. After the 9 day period, melamine was withdrawn by placing the cows on the control pasture that did not receive melamine contaminated fertilization for another 7 days. During these 16 days, milk was collected twice a day, viz. during the morning and afternoon milkings. Milk samples of each cow were sub-divided into two samples, one was preserved with potassium dichromate and analysed for milk composition and the other was frozen until analysed for melamine by LC-MS/MS. For the duration of the trial, melamine containing milk was destroyed in order to prevent it from contaminating milk collected from the rest of the herd. Results from the analysis for melamine confirmed that melamine was transferred from melamine fertilized pasture to milk. In this study, it took 6 days from melamine withdrawal for melamine to reach undetectable levels in the milk. It was also found that the melamine fertilized pasture did not have any significant effect on the average milk production and milk composition of the cows. The aim of the study was met and it was confirmed that melamine can be transferred from fertilizer to the soil, to the pasture and to the milk of cows grazing these pastures.

Uittreksel

Titel	:	Melamien, van kunsmis tot weiding tot melk.
Kandidaat	:	Dawn Dorothy Botha
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Die doel van hierdie studie was om die tempo van oordrag van melamien as bemestingstof na Kikuyu weiding te bepaal, asook om te bepaal of melamien oorgedra sal word vanaf die bemeste weiding na melk. Drie proewe is uitgevoer wat ‘n potplant loodsproef, ‘n toegepaste weidings proef en ‘n melkproduksie proef ingesluit het.

Melamien is ’n kommersieël-beskikbare industriële chemikalieë met ’n hoë stikstof inhoud. Groot hoeveelhede van melamien-afval kan soms in kunsmis ingesluit word vir die bemesting van gewasse en weidings, weens die hoë stikstof inhoud van melamien. ’n Potplant loodsproef met Kikuyu is uitgevoer om te bepaal of melamien vanaf die grond deur die plantmateriaal geabsorbeer word. Melamienbemesting is in die vorm van vervalste Chinese mieliegluten 60 toegedien teen ’n hoeveelheid gelykstaande aan 8.8 kg melamien/ha. Die resultate van hierdie proef het getoon dat melamien wel deur die plantmateriaal geabsorbeer is en 7 dae nadat bemesting toegedien is, was die konsentrasie van melamien in die gras 228 of mg/kg.

’n Toegepaste weidingstudie is uitgevoer waar drie kampe van 0.3 ha elk gebruik is. Een van die kampe het as ’n kontrole gedien en het stikstof bemesting in die vorm van KAN teen 40 kg N/ha ontvang. Die ander twee kampe het KAN bemesting ontvang waar 10% (Behandeling 1) en 20% (Behandeling 2) van die KAN-N deur melamien-N vervang is. Al drie kampe het ook fosfaatbemesting in die vorm van Enkel Superfosfaat ontvang teen 20 kg P/ha, asook KCl kunsmis teen 50 kg K/ha. Weidingmonsters is eenmaal per week op dieselfde plek in elke kamp geneem vir 10 weke nadat bemesting toegedien is geneem. Monsters is gedroog en daarna fyngemaal voordat dit vir melamieninhoud geanaliseer is met behulp van LC-MS/MS. Die aanvanklike melamienkonsentrasie in die gras van Behandeling 2 was hoër as die in die gras van Behandeling 1. Die tempo waarteen die melamienkonsentrasie in die plant materiaal afgeneem het, was baie dieselfde vir Behandelings 1 en Behandeling 2. Dit het ongeveer 10 weke geneem voordat die melamien in die gras nie-waarneembare vlakke bereik het. Die gevolgtrekking is gemaak dat melamien wat in die vorm van kunsmis toegedien word, as sodanig uit die grond geabsorbeer word deur die gras.

Vir die melkproduksiestudie is agtien lakterede Holsteinkoeie, 60 ± 5.1 (SE) dae in melk, met ‘n daaglikse melkproduksie van 36.5 ± 2.0 (SE) kg/dag en ‘n liggaamsmassa van 609 ± 12.8 (SE) kg, volgens hul melkproduksie gerangskik en ewekansig in drie groepe van ses koeie elk ingedeel. Die groepe is daarna ewekansig aan die drie kampe, wat in die toegepaste weidingstudie gebruik is toegedien. Die koeie is vir 9 dae op die melamienbemeste weidings gehou, waartydens hulle toegelaat is om vir ongeveer 10 ure elke dag te wei. Na die tydperk van 9 d, is die koeie vanaf die melamienbemeste weidings onttrek deur hulle op die kontroleweiding te plaas vir ’n verdere 7 dae. Gedurende hierdie 16 dae is melkmonsters tweekeer per dag geneem, tydens die oggend- en die middagmelkings. Die melkmonsters van elke koei is vervolgens onderverdeel in twee monsters, waarvan een met kaliumdichromaat gepreserveer is vir die beplaing van melksamestelling, terwyl die ander een gevries is totdat dit later vir melamien inhoud ontleed is met behulp van LC-MS/MS. Melk van die koeie wat op die melamienweidings gewei het, is weggegooi om te voorkom dat melk van die res van die kudde gekontamineer kon word. Die resultate van die melamienanalises het getoon dat melamien oorgedra word vanaf die melamienbemeste weiding na die melk. Tydens hierdie studie het dit 6 dae geneem vandat koeie vanaf die melamienbemeste weidings onttrek is, totdat die melkmelamien nie-bepaalbare vlakke bereik het. Daar is bevind dat melamienbemeste weidings geen betekenisvolle uitwerking op die gemiddelde produksie en samestelling van die melk gehad het nie. Die doel van hierdie studie is bereik en daar is getoon dat melamien vanaf kunsmis na die grond, na die gras en na die melk oorgedra kan word wanneer koeie op weidings geplaas word wat melamienbemeste kunsmis ontvang het.

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CHAPTER 1

GENERAL INTRODUCTION

Milk and other dairy products play a very important role in the daily nutrition of people everywhere. It is the main food source for infants, in the form of powdered infant formula or liquid milk. Consumers in South Africa bought 2.50 billion litres of milk and milk related products during 2009 (Milk Producers Organization, 2010). Milk contributes to the economic growth of a country by means of exportation of various dairy and other products manufactured from dairy products. Strict quality control measures need to be in place to ensure that the milk and other dairy products that are available in stores and supermarkets are safe for human consumption. Milk needs to be free not only from pathogens, but also from other chemical contaminants.

A well-documented case where quality control was not up to standard was the melamine milk scandal in China during 2008. Melamine ($C_3H_6N_6$) is a chemical used in the production of many household items such as plastic kitchenware, but also industrial products such as flame-retardants, glues and dyes. Melamine itself is not considered toxic, but the adulteration of dairy products with large amounts thereof can lead to renal failure and in the worst cases, even death (Cheng *et al.*, 2010). Melamine is relatively high in nitrogen (N), around 670 g/kg N on a molecular weight basis (Merck, 1987). The protein content of a foodstuff or raw material is determined through the amount of N the product contains, as protein is made up of N. Most tests used for the determination of protein do not distinguish between true protein and non-protein nitrogen (NPN). Melamine adulterated milk will thus have a high protein value, but it is in fact non-protein nitrogen from the melamine, which gives this false value. Adding melamine to milk was considered a very profitable practice. The gain from adulterating 1 billion kg of milk was found to be around US$ 87 million (Xiu & Klein, 2010). Dairy farmers, as well as other parties involved in the supply chain aimed for an increased profit, while at the same time reducing input costs. Milk which should have been rejected due to its poor or sub-standard quality was being sold for high prices. Watered down milk was mixed with melamine to restore the apparent protein content and then sold to consumers. This contaminated milk was used to produce the infant formula that caused renal-related illnesses among babies and the death of 6 infants in the mainland of China (Xiu & Klein, 2010).

Deliberate adulteration of milk is not always how melamine ends up in milk. Numerous other ways may cause milk to contain melamine. A recently researched pathway is the transfer of melamine from contaminated animal feed to milk. A study done by the University of Stellenbosch, South Africa, confirmed that melamine can be detected in the milk of dairy cattle after receiving melamine adulterated feed. The feed used in this trial contained a melamine-contaminated source of maize

Gluten 60. This Gluten 60 was of the same batch purchased from China that caused the pet food recall and the death and illnesses of thousands of pets in South Africa and other parts of the world. The Gluten 60 contained, amongst other things, melamine, cyanuric acid, urea, and other metabolites of melamine (Cruywagen *et al.*, 2009). Another study of the same nature, using dairy goats, was recently done by Baynes *et al.* (2010), who found that after a single oral dose of melamine at a rate of 40 mg/kg BW (body weight), melamine can be detected at levels well above the redeemed safe level of 1.00 mg/kg for infant formula for approximately 3 days after dosing in the milk of dairy goats. The study showed that melamine has a 3 – 6 times longer half-life in small ruminants than in monogastric species such as pigs and rodents. The reason for the extended half-life is unclear, but one possible explanation is that the rumen acts as an additional compartment, thereby increasing the apparent volume of distribution of the melamine. When comparing the studies of Baynes *et al.* (2010) to Cruywagen *et al*. (2009) one can conclude that melamine is eliminated in a shorter time in dairy cattle than in dairy goats. This can be contributed to the fact that milk yield for cattle is higher than for goats.

The Code of Federal Regulations (CFR) in the United States has approved cyanuric acid, a metabolite of melamine, as a source of NPN for cattle. Cyanuric acid together with melamine was found to be the cause of pets becoming ill from contaminated pet food, as already mentioned. It is not yet clear if cyanuric acid is converted to melamine in the rumen, or if it has any adverse effects on the health of ruminants *per se* (Baynes *et al.,* 2010).

Another possible pathway through which melamine can enter milk is by means of Cyromazine. This chemical is used in the agricultural industry mainly as an insecticide to prevent the hatching of flies from manure and as a foliar spray pesticide for crops. One of the metabolites of cyromazine is melamine. Melamine can potentially find its way into animal products by the ingestion of cyromazine that has been sprayed on crops or by the actual administering of the cyromazine drug to the animals (Liu *et al.*, 2010).

Melamine is also used as an agricultural fertilizer for crops and pastures. Fertilization of pastures and crops play an important role in ensuring that the soil in which these plants grow has the correct amount of nutrients available for the plants to grow. Some of these plants will in turn form part of animal feed either directly such as the pasture, or indirectly as part of processed animal feeds. Little is known on the rate of transfer from the melamine-fertilized soil to the plants. It is unclear if plants absorb melamine from the soil via the root system, or via the leaves of the plant. No previous trials have been conducted to determine the role that melamine as a fertilizer plays in the contamination of animal products with melamine.

The aim of this study was to determine the rate of transfer from melamine as fertilizer to kikuyu pastures and if melamine would be transferred from the fertilized pasture to milk of dairy cattle.

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) or $C_3H_6N_6$ is a chemical compound widely used in the manufacturing of everyday household products such as plastic kitchenware, utensils, glues and laminated flooring. It is also a component of laminated plywood, fireproof paint and industrial cleansers (Ingelfinger, 2008). In addition, melamine is a chemical component of the dye, pigment yellow and many commercially available fertilizers (Yang *et al*., 2009). Another name for melamine is tripolycyanamide (Chan *et al*., 2008). When combined with formaldehyde, melamine resin is produced which is a highly durable thermosetting plastic product (Yang *et al*., 2009).

The United States and other Western countries also produce large quantities of melamine, much the same as China (Ingelfinger, 2008).The melamine in plastic containers can eventually migrate to the food held in the container, but because of the minute quantities, the levels are not considered to be toxic (Ingelfinger, 2008).

During the manufacturing of melamine, several compounds can be formed which are usually found in association with melamine. These compounds include ammeline (4,6-diamino-5H-1,3,5-triazine-2-one), ammelide (6-amino-1H-1,3,5-triazine-2,4-dione), melem, melam, melon and cyanuric acid (1,3,5-triazine-2,4,6-trione) *(Xie et al*., 2009). Cyanuric acid is structurally similar to melamine and is used as a stabilizer to prevent the breakdown of hypochloric acid, which is used in swimming pools, by light (Downes *et al.*, 1984; Puschner *et al*., 2007).

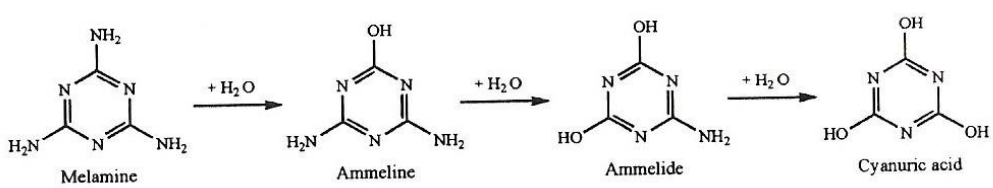

Figure 1 Structures and formation of ammeline, ammelide and cyanuric acid by alkaline hydrolysis of melamine (Muñiz-Valencia *et al*., 2008).

2.2 Melamine as adulterant

Adulteration and the accidental contamination of foods has been a reality throughout time. Globalization causes another problem. Contaminated food can spread throughout the world in a matter of days. An example of this is the horrifying story of melamine adulterated baby formula in China. At least 6 infants died and somewhat 50 000 were treated in hospitals. The infant formula had far-reaching effects. Children in Vietnam, Taiwan, Singapore and other parts of Asia also paid the price (Ingelfinger, 2008).

The question posed is then: why would one deliberately add compounds of no nutritional value, such as melamine to foods? Increasing the apparent protein content at a low cost is one of the reasons to add melamine to milk. The most important reason to adulterate milk and other products is of economic motivation, in other words, manufacturers will obtain a higher price for a below-standard product (Yang *et al*., 2009). The amount of nitrogen in foods or animal feeds is used as a tool to determine the protein content of the particular food. Thus, the higher the nitrogen content, the higher the apparent protein content. When determining the protein content of food or feeds, no distinction can be made between true protein and non-protein nitrogen sources (Ingelfinger, 2008). One of the most commonly used tests to determine protein content, the Kjeldahl test for instance, measures the nitrogen content, but does not differentiate between true protein and non-protein nitrogen (Yang *et al*., 2009). The protein components used in feeds are more commonly at risk of being adulterated than other components, as it is the nitrogen part that can be falsely increased by the addition of melamine and its metabolites. Raw materials with high non-protein nitrogen content and low amino acid content should be regarded as suspicious and analyzed for contaminants and adulterations (Puschner *et al*., 2007). As Melamine typically contains 670 g/kg nitrogen on a molecular basis (Merck, 1987), it is an attractive substance for the adulteration of feed sources.

2.3 Melamine in plants

The industrial use of melamine produces millions of tons of waste. The melamine waste, which is high in nitrogen, is often used as a crop fertilizer in many countries (Ingelfinger, 2008). China is not only the greatest consumer of chemical fertilizers, but it also uses the most pesticides. This makes the possible application of melamine as a fertilizer more common than in countries that place restrictions on the use of fertilizers such as the European Union (EU) for instance (Sternfeld, 2009). From as early as the 1950's, melamine has been applied as a crop fertilizer because of its high nitrogen content (Hauck & Stephenson, 1964; Puschner *et al*., 2007; Yang *et al*., 2009). It is suggested that the melamine is absorbed into the soil and then taken up by the plant (Ingelfinger, 2008).

The triazine pesticide, cyromazine (N-cyclopropyl-1,3,5-triazine-2,4,6-triamine) is used in agricultural practices to inhibit insect growth and to control flies on fruits and vegetables and field crops (Sancho *et al*., 2005; Muñiz-Valencia *et al*., 2008). Cyromazine shows a strong translaminar effect when it is applied to the leaves of plants. It is taken up by the roots and translocated acropetally when applied to the soil (Hartley & Kidd, 1983). When cyromazine is exposed to light or extreme temperatures, it can be degraded to form melamine. It can also be environmentally degraded, or degraded by dealkylation reactions in normal plant metabolism. This is another way in which melamine can end up in plant material without being directly applied to the plant itself or to the soil (Sancho *et al*., 2005; Muñiz-Valencia *et al*., 2008).

From the trial conducted by Illinois workers, the conclusion that had been made was that melamine in combination with urea was a poor fertilizer for domestic lawns. The colour response of the turf grass used in the trial was slow and the growth response was low. The reason for the observations made was that melamine was found to be a slow-release fertilizer. In the lawn care industry, a quick response to colour is required in order for the product to be acceptable to the consumer (Wehner & Martin, 1989).

The *in vitro* degradation of melamine to ammonia by the partial alkaline hydrolysis of the amino groups or by strong acids is a relatively easy process. In contrast, the biochemical degradation of melamine in soils takes place at a much slower rate. Workers postulated that this slow degradation rate in soils could be due to the molecule having a symmetrical resonating structure. Symmetrical molecules tend to be more stable, which makes them more difficult to degrade biochemically (Hauck & Stephenson, 1964).

A series of tests were conducted to ascertain the value of melamine as a potential nitrogen source for plants. One of the results obtained showed that about 1% of melamine nitrogen was converted to nitrate in comparison to the 80% of ammonium sulphate nitrogen converted to nitrate. These tests once again confirmed that melamine is a slow-release nitrogen fertilizer (Scholl *et al.*, 1937). Studies done during 1987 concluded that melamine would not be a preferred fertilizer for grasses, primarily due to its slow release of nitrogen into the soil. It can however be used together with other nitrogen sources such as urea or biuret (Mosdell *et al.*, 1987).

2.4 Melamine in human health

Melamine can pose a threat to human health in many ways. It causes irritation to the eye and skin mucous membranes when inhaled by factory workers and other people exposed to melamine powder (Suchy *et al*., 2009; Yang *et al*., 2009). Melamine also has a negative influence on the digestive tract and in turn other organs of the human body. Humans are unable to completely degrade melamine and the excretion thereof takes place *via* the urine after it is absorbed from the gastrointestinal tract (Filigenzi *et al.*, 2008; Langman, 2009). Within 24 hours most (>98%) of the orally administered cyanuric acid had been excreted via the urine in human test subjects (Puschner *et al*., 2007). It has been known for a long time that melamine and melamine derivatives have the potential to be toxic. The rate at which toxicity occurs however, has not yet been determined. The World Health Organization (WHO) has set a tolerable daily intake (TDI) of 0.2 mg/kg of body weight for melamine and 1.5 mg/kg of body weight for cyanuric acid (Ingelfinger, 2008). The current limit for melamine in food, which had been adopted by many countries, including South Africa, is 2.5 mg/kg. These values were calculated based on ingestion by a person weighing 60 kg. There is unfortunately not enough human data available to ensure exact figures (Ingelfinger, 2008). The US Food and Drug Administration (FDA) has set the TDI at 0.63 mg/kg body weight according to Chan & Lai (2009). TDI is defined as “the estimated maximum amount of an agent to which individuals in the population may be exposed daily over their lifetimes without appreciable health risk” (Hsieh *et al*., 2009). The safety levels for adults with respect to dairy products cannot be applied to children: the reasons being that young children are especially vulnerable to melamine toxicity, because their diets consist mainly of dairy- and dairy derived products and because children have a much lower glomerular filtration rate (GFR) when compared to adults (Hsieh *et al*., 2009). Small children and babies are at a higher risk when consuming melamine-tainted products. The reason for this is that their organs are not yet fully developed which makes them extra vulnerable to toxic compounds (Chan *et al*., 2008).

Melamine has been found to be transmitted to animal tissues, milk and eggs and thus poses a larger threat to the global community, especially young children. High doses of melamine caused renal failure in the affected infants in China. Those that died were found to be treated too late, as infants and young children cannot say that they feel ill. This is also true in the case of animals (Ingelfinger, 2008).

The first reports of melamine toxicity in humans appeared on September 11, 2008. Renal failure and kidney stones were found in young children and babies after they had consumed melamine tainted baby formula. One of the main culprits in the melamine scandal was the Sanlu group in

China, although other companies were also found to be involved (Lai, 2008). Children diagnosed with urinary calculi in China in 2008 had been consuming tainted infant formulae that had melamine concentrations as high as 2563 mg/kg present (Zhang *et al*., 2009). Children do not normally experience renal associated problems as these conditions are more commonly associated with adult (> 25 years old) individuals (Lam *et al*., 2009).

Kidney stones found in the children from the Mainland of China were sand-like and loose and could be passed out by the patient after alkalinisation of the urine and hydration were applied. Figure 2 illustrates the type of kidney stone found in one infant. In some severe cases, surgery was required, which included percutaneous kidney drainage, lithotripsy and cyatoscopic catheterisation into the urethras of the patients (Lai, 2008).

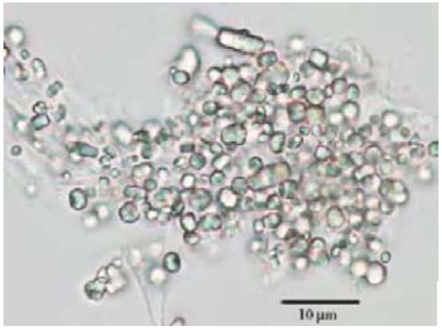

Figure 2 Melamine + cyanuric acid crystals found in the urinary sediment from an affected infant (Langman *et al*., 2009).

Because melamine kidney stones are radiolucent, they are not always visible on x-rays. Scanning with ultrasound is a much more successful way to detect kidney stones. The negative aspect of this is that ultrasound can only detect kidney stones in the patient and not other effects in the kidneys caused by melamine such as tubulointerstistial nephritis and tubular crystals (Lai, 2008). A simple urine test was developed to test for melamine in the urine, but further studies are required to investigate the clinical application thereof in melamine-related kidney disorders in human patients (Lai, 2008). Table 1 summarises some of the clinical symptoms observed in children who had consumed tainted milk.

Table 1 Common symptoms and signs according to Langman *et al*. (2009) in infants experiencing urinary calculi.

• Unexplained crying during urination
• Vomiting
• Stones discharged while urinating
• Unexplained fever caused by urinary tract infection
• Bacteraemia caused by urinary stasis
• Acute obstructive oligo-anuric kidney failure
• Microscopic and macroscopic hematuria
• High blood pressure
• Oedema
• Unexplained flank pain

The treatment of patients by means of alkalinisation of the urine has been successful in the case of uric acid being present in the kidney stones. This alkalinisation will then essentially dissolve the kidney stones and ensure that the remains be passed out by the patient. In some cases, melamine forms a complex with cyanuric acid. This complex will dissolve better in more acidic urine. It is thus essential to determine the contents of the kidney stones in some way before manipulating the pH of the urine to ensure that the stones be successfully dissolved and not to worsen the condition in the patient (Lai, 2008).

Premature born babies were found to be more susceptible to kidney stone formation than full term babies (Langman, 2009). The reason for this condition is that the inhibitors of stone formation are excreted at much lower rates than normal. This condition may persist throughout the infant's life. These premature born babies are thus at a higher risk of developing renal failure and kidney stones when consuming melamine-tainted milk products. This is just another one of the many reasons why melamine in foods and feeds should be completely eradicated as to avoid such accidental intoxications, which may lead to the death of a child (Langman, 2009). The exact way in which humans respond to melamine toxicity is not clear and requires further investigations (Zhang *et al*., 2009).

There have also been speculations that melamine poses a threat to the health of unborn foetuses. Melamine crystals are smaller than the placental blood vessels, which causes them to be passed on from the mother to the foetus. Prolonged periods of exposure to melamine might be the cause of urinary tract disorders and abnormalities of the kidneys as well as the urinary tract of the unborn foetus. This might take place when the mother is exposed to high doses of melamine in her diet during pregnancy. Earlier results have shown that melamine is a likely cause of foetal abnormalities and even death. It is unknown if melamine affects the foetus in a mutagenic or teratogenic manner (Generoso *et al.*, 1988; Wiwanitkit, 2009).

A study done by Lam *et al.* (2009) showed that the pathogenesis of melamine associated renal stone disease could be different in humans from animals. The melamine crystals found in human subjects were different from those found in animals in 2007, which were from a melamine-cyanurate complex. The crystals in humans were globular- to almost flattened in shape with a soft consistency. The melamine-cyanurate crystals in the pets were of a spherical shape and a hard consistency (Lam *et al*., 2009). The study suggested that in contrast to the way in which renal stones formed in pets consuming tainted feed, cyanuric acid does not play the same role in stone formation in humans as it did in the pets. This might explain the difference in the morphology of the renal stones found in the human subjects (Lam *et al*., 2009). When Figures 3 and 4 are compared, one can clearly observe the differences in stone formation. Human subjects (Figure 3) will have an oval shaped, soft stone. Note the golden brown colour of the stone. Figure 4 shows spoke-like crystals which are observed in animals. These are hard crystals.

Figure 3 An optical microscope image of urinary bladder stones formed in the human subjects due to melamine ingestion (Grases *et al*., 2009).

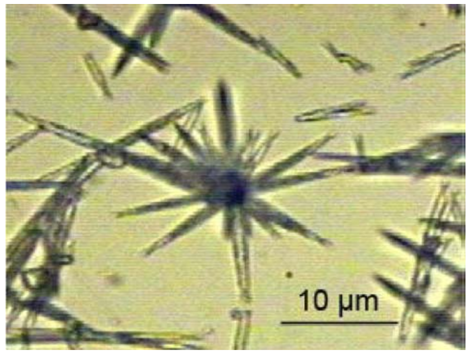

Figure 4 Spoke-like crystals found in animal subjects when melamine and cyanuric acid combine (He *et al*., 2008).

2.5 Melamine in foods and feeds

The nitrogen content of food or feeds can be increased by the addition of melamine and even cyanuric acid (Zhang *et al*., 2009). Melamine is not, in spite of its high nitrogen content, intended for animal and even less so, human consumption (Langman *et al*., 2009). A trial was conducted in 2008 at Stellenbosch University, South Africa, to determine if melamine from feed is carried over to the milk. Results that were obtained confirmed the hypothesis. When cows consumed the melamine adulterated feed, traces of melamine appeared in their milk as early as 8 hours after the first consumption. When cows were withdrawn from the melamine diet, the melamine persisted in the milk for up to 7 days afterwards (Cruywagen *et al*., 2009).

There are numerous ways in which melamine can eventually find its way into food, some of which are everyday practices to package and process foods. Melamine has been reported to appear in trace amounts in some foods due to the possible migration of melamine from the plastic packaging, which is made of melamine resin (He *et al*., 2008). Also, speculations have been made that melamine could also be present in trace amounts when cyromazine, which is used as a spray-on pesticide in the production of fruits and vegetables, is not completely removed before foods are processed and packaged (He *et al*., 2008).

Melamine or cyanuric acid was not found in dairy products alone, but also in some biscuits and chocolates, which might had tainted milk or milk powder as ingredient. The European Union (EU) has set up legislation which stipulates that all products containing melamine above or equal to 2.5 mg/kg shall be immediately destroyed (Puschner *et al*., 2007). Some researchers claim that contaminants in livestock feeds do not pose a threat to human health. This cannot be blindly accepted as melamine can be carried over from the feed to eggs, milk and meat (Puschner *et al*., 2007).

2.6 Melamine and animal health

Melamine does not have a significant acute oral toxicity, but the feeding of large quantities thereof causes kidney stones in animals (Chan *et al*., 2008). During 2007, numerous pets around the world died after consuming pet food, which contained melamine adulterated maize gluten (Cruywagen *et al*., 2009). Acute toxicity of melamine can lead to renal failure, whilst chronic toxicity can lead to kidney and/or bladder stones (Puschner *et al*., 2007). Workers have also hypothesized that melamine could have negative effects on the reproductive system of animals and have teratogenic effects on unborn foetuses (Puschner *et al*., 2007). A teratogen is any substance, organism or process that causes malformations in the foetus. Acute toxicity of cyanuric acid can lead to hyperplasia and necrosis of the kidneys, fibrosis, infiltration of the neutrophiles and dilatation of kidney tubules (Puschner *et al*., 2007). An outbreak of kidney failure in companion animals throughout Asia during 2004 was caused by the consumption of specific tainted pet foods. A similar outbreak occurred in North America during 2007 among companion animals. The same toxicological and histological finding was observed in 2004 and 2007 outbreaks (Xie *et al*., 2010). The renal tubules of pets where damaged when they consumed feed tainted with melamine. This was because of the insoluble crystal that formed in the kidneys and renal tubules (Brown *et al.*, 2007; Puschner *et al.*, 2007; Zhang *et al*., 2009). The insoluble crystal found in these domestic pets, occurred mostly in the distal tubule (Langman *et al*., 2009). These insoluble crystals can serve as a possible biomarker for feed contaminations (He *et al*., 2008).

During 1966, a feeding trial was conducted at Onderstepoort Veterinary Institute, South Africa to ascertain if melamine could be used as a source of NPN in ruminant diets containing poor quality roughage (MacKenzie, 1966). Melamine was added at 9.88 or 19.6 g/sheep per day to the different diets fed. It was concluded that melamine was not an effective source of NPN for sheep as it caused a decline in the voluntary intake of roughage. The author did not investigate the toxicological effects of melamine in his trial (MacKenzie, 1966). Newton & Utley (1978) also did a trial to determine the value of melamine as a source of non-protein nitrogen for ruminants. They concluded that melamine would not be a suitable source of nitrogen for microbial synthesis in the rumen because melamine is poorly hydrolyzed in the rumen. This was apparent in the low ammonia nitrogen content found in the rumen liquor of the animals receiving melamine in their diets.

A toxicology trial was later conducted also at Onderstepoort Veterinary Institute, South Africa to investigate specifically the formation of crystalluria in sheep that received melamine (Clark, 1966). During the trial, a number of experiments were conducted to establish at which level melamine becomes toxic to the animal (Clark, 1966). In the first experiment of Clark (1966), a sheep wether of 46 kg was given a single oral dose containing 100 g of melamine. Ten days after this dosage of melamine was administered, the sheep stood with an arched back, which is a typical symptom of anuresis. It also presented signs of severe anorexia. Due to ethical reasons, the sheep was euthanized the following day. During the post-mortem, crystals were found inside the kidney tubules (Clark, 1966). In the second experiment of the same trial, a 37 kg wether was dosed for 6 consecutive days with 50 g of melamine per day. On day 5, symptoms of anorexia and anuresis were observed. Only a small amount of bloody urine was passed. On day 7, the sheep had died. The post-mortem found the results to be the same as in the first experiment (Clark, 1966). Four other experiments were conducted, all with varying levels of melamine administration over different periods of time. All of the experiments, except one, showed exactly the same symptoms and post-mortem results as the first 2 experiments. In the experiment with differing results, the sheep were fed melamine at a dosage of 7 g/sheep per day. The sheep showed no ill effects due to the treatment. Melamine could thus be considered to be safe when dosed at 7 g/day or less. From the preceding evidence, it can thus be concluded that melamine is equally toxic at high level single doses and lower level doses over longer periods of time (Clark, 1966). During the trial, other interesting observations were also made by the author. The blood pH was elevated to 8.5 – 9.0. No toxic effects due to melamine were found in the liver and the ruminal pH or ruminal motility (Clark, 1966). One has to keep in mind that the trial was conducted in 1966 and further investigations are needed to determine the true effects of melamine on other organs of the body.

Melamine and the melamine-derivative, cyanuric acid, were identified as the cause of the renal failures, and pet food all over North America and various other countries was recalled (Xie *et al*., 2010). The symptoms, which were commonly observed in the pets that consumed tainted feed, included vomiting, lethargy, polyuria and anorexia (Puschner *et al*., 2007). Chronic histological changes characterized by inflammation and interstitial fibrosis were found in others. Owners of some cats observed refusal to eat, polyuria, vomiting, lethargy and excessive thirst before they were taken to a veterinarian for treatment. When the vomitus, urine, kidneys and cat feed were toxicologically sampled, traces of melamine and cyanuric acid were found. When post-mortems were performed on some of the animals that died from acute renal failure, crystals where found in the kidney tubules (Puschner *et al*., 2007). Figure 5 shows microscopic images of damaged kidney tubules observed in a dog that had consumed melamine tainted pet food.

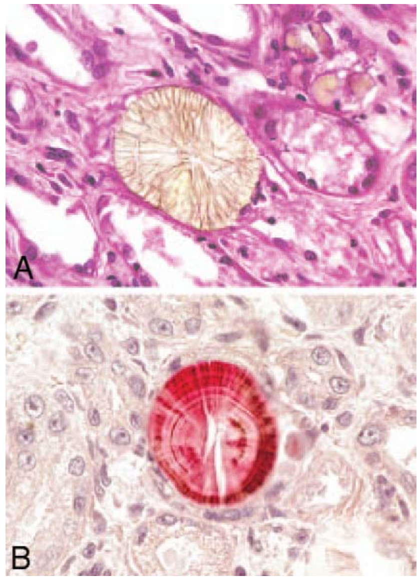

Figure 5 Photomicrographs of melamine-containing crystals within the renal tubules of necropsy kidney from a dog with a history of ingesting melamine adulterated pet food.

A. Oil-red and B.Original magnification (x200) (Lewin-Smith *et al*., 2009)

Wheat gluten and rice protein were adulterated by the addition of melamine, which increased the apparent protein content of these ingredients (Xie *et al*., 2010). During 2007, some pork producers in the USA fed melamine tainted pet food to their pigs. Approximately 6000 pigs where immediately quarantined and where due to be euthanized by the United States Department of Agriculture. Seven states, which included Utah, South Carolina, North Carolina, Oklahoma, New York, Kansas and California, where affected. The FDA stressed that farmers should keep records that are more accurate and make sure that the feeds obtained are from a reliable, melamine-free source (Vansickle, 2007).

A trial conducted on cats by Puschner *et al*. (2007), investigated the toxicity of the combination of melamine and cyanuric acid. The experimental cats began to vomit and developed anorexia and slight depression approximately 12 hours after receiving a dosage of melamine together with cyanuric acid. Both melamine and cyanuric acid was given at the same rate of 32 mg/kg body weight, 121 mg/kg body weight or 181 mg/kg body weight to the three experimental cats (Puschner *et al*., 2007). Whether or not insoluble complexes would form between melamine and cyanuric acid would largely depend on the pH of the urine. Cat and dog urine tends to have a lower, more acidic pH, which places them at a higher risk of developing kidney- and bladder stones than other animals that might have more alkaline urine. During the trial, it was shown that a single oral dosage of melamine and cyanuric acid at a concentration of 32 mg/kg body weight was sufficient to have caused acute renal failure followed by subsequent death of the experimental animals (Puschner *et al*., 2007).

Many contrasting results have been found in this field. Some workers have found that, on its own, melamine appears to be non-toxic. However, when combined with cyanuric acid, insoluble melamine cyanurate crystals will form (Grases *et al*., 2009). This statement has only been experimentally tested in animals, and would not necessarily apply to humans.

An experiment conducted by Xie *et al*., showed that when melamine is fed in high doses or when it is fed in combination with cyanuric acid, it results in the formation of renal stones. These "stones" are believed to have been the main cause of renal failure in the infants in China as well as the cats and dogs around the world. Xie *et al*. (2010) found that the pathways most significantly affected by melamine are those of amino acid metabolism, some of which include polyamine, tryptophan and tyrosine metabolism. They also found that the gut microflora was altered in structure. Elevated levels of urinary trimethylamine oxide (TMAO) in the experimental rats indicated renal papillary damage. This TMAO also served as a marker to indicate renal damage in further experiments conducted (Xie *et al*., 2010). A combination of melamine and cyanuric acid, at a rate of 50 mg/kg body weight each, yielded the same renal damage in the experimental rats, as did the high doses of melamine, at a rate of 600 mg/kg body weight. It can be concluded that melamine in conjunction with cyanuric acid is equally dangerous to animals as it affects the same amino acid metabolism pathways as high doses of melamine does (Xie *et al*., 2010).

2.7 Analysing for melamine

Animal feeds and the components thereof are complex matrixes and this often makes the screening for contaminants and residues a difficult process (Muñiz-Valencia *et al*., 2008) . The matrixes vary significantly and the exact composition of the matrix is not always known to the analysis laboratory. Feeds are often a combination of oil seeds, roughages, cereal grains, fats and other by-products. Preservatives, minerals and vitamin premixes are also added to feeds. Muñiz-Valencia *et al*. (2008) suggested that a concentration of 0.05% melamine or cyanuric acid does not have a significant increasing effect on the apparent protein content of the feed, but when the concentration exceeds 2%, the melamine and/or cyanuric acid would give a bitter taste to the feed, making it unpalatable to the animals. Feed refusal by the animals is often one of the first indications that there is some or other thing wrong with the ingredients of the feed (Muñiz-Valencia *et al*., 2008)

The following methods can be used for analysis of melamine and is based on information provided by the World Health Organization (2008):

2.7.1 LC-MS/MS (Liquid chromatography tandem mass spectrometry)

Liquid chromatography tandem mass spectrometry is considered the most dependable method to use during low level quantification of melamine in many types of samples. It is highly selective and sensitive over a wide range of raw materials. A mixture of water and acetonitrile is used to extract the melamine from the samples. Liquid extraction is used to clean the samples after which a solid phase extraction follows. Highly sophisticated equipment is needed for this procedure to be carried out. Reliable quantification at a low level is made possible by isotopically labelled standards (World Health Organization, 2008).

2.7.2 LC-MS (Liquid chromatography mass spectrometry)

This method performs comparatively well to the LC-MS/MS method. It is also a highly selective quantification method. Careful evaluation of interference from the sample is needed, but not many compounds will interfere as melamine has a unique isotopic pattern and a protonated molecular mass. This method will give a reliable quantification if the sample is cleaned up by means of ion exchange (World Health Organization, 2008). Another advantage of this method is that the samples are easy to prepare and that no organic modifier is used (Muñiz-Valencia *et al*., 2008).

2.7.3 GC-MS (Gas chromatography mass spectrometry)

Preparation of the sample is very labour intensive because before the melamine can be injected into the GC-MS, it first has to be derivatized. If the procedure is performed correctly and on a well-kept system, the results will be highly selective and sensitive. A combination of diethylamine, acetonitrile and water is used to extract the melamine from the samples. Before analysis, the analytes are converted into trimethylsilyl derivates. This method can be used to analyse a wide range of samples (World Health Organization, 2008).

2.7.4 (HP)LC-UV

Before the samples are analyzed by the ion-pair HPLC, they are extracted with a mixture of water and acetonitrile. A lot of interference can be expected with some complex samples such as biscuits and chocolate bars. This is mainly due to the interfering compounds of these foods that will also absorb UV light at 240 nm. For each individual food matrix and intensive ratification by a mass spectrometric based technique is required for the development of an LC-UV. A particular sample preparation procedure may also be required for each of the different food matrices (World Health Organization, 2008).

2.7.5 ELISA (Enzyme-linked immunosorbent assay)

This quick method is presently used on a large scale, particularly in the dairy industry. Melamine can be detected in feeds, milk and milk powder by this technique. Melamine is extracted from the samples by means of sonication or by counter current. The extract is then pipetted into antibody-coated micro wells. The amount of melamine is established by means of a colour reaction and the optical density is measured by a micro-plate reader. The upper level of detection is presently set at around 1500 ppb and the lower level is set at around 60 ppb (World Health Organization, 2008).

2.7.6 Rapid melamine detection kits

These tests are a quick and inexpensive way to detect melamine in milk. The tests are based on a dipstick principle. “Melaminesensor”, was developed by Unisensor and remains one of the most sensitive and rapid tests of this kind and is able to detect melamine in milk at a level of 250 ppb (or 0.25 mg/kg) in 5 minutes. Seven of these kits are either already commercially available, or soon will be. Melaminesensor is made up of reagents, a set of micro-wells and the sensor dipsticks. A Readsensor can be used in addition to the visual interpretation to give more accurate results. First, the reagents are allowed to reach room temperature before used. Next, 200 µL of milk is pipetted into the micro-wells and incubated at 40°C for 1 minute. After incubation, the dipsticks are inserted into the micro-wells for 4 minutes, if a quick protocol is required, or 8 minutes, if a sensitive protocol is required. The dipsticks are removed from the micro-wells and their filters removed. Results can now be visually interpreted or placed into the Readsensor for a more sensitive reading. The results can be visually interpreted as follows: test □ control yields a negative result and < 250 ppb melamine can be present; test = control yields a low positive result and ~ = 250 ppb melamine can be present; test = 0 < control yields a positive result and >1000 ppb melamine can be present. Instrumentally, the results can be in a range of 125 – 1000 ppb of melamine. Melaminesensor is compatible with pet foods, but this type of test is not yet developed (http://www.primepharma.co.za/products/lab-equipment/melamine-quick-test/ Accessed 2010-09-12).

2.8 Conclusion

One should always keep in mind that melamine does not belong in feeds and foodstuffs at any level (He *et al*., 2008). Melamine and cyanuric acid form insoluble crystals in the microtubules of kidneys and this melamine-cyanurate complex is much more toxic than either melamine of cyanuric acid on their own (Puschner *et al*., 2007). Large-scale damage had been caused by melamine adulterations in the previous years. Animal feeds as well as human food products have been affected. The most effective manner in which adulterations can be prevented is to ensure that a good quality control program is put into place. Feed companies must keep adequate records of their raw materials and must ensure that the raw materials are sourced from reliable manufacturers. It is generally a good practice to submit all newly acquired raw materials to tests before the manufacturing of feed commences. Melamine has not only had a major impact on the health of many humans and animals, but it has also severely crippled the dairy industry and also in many instances part of the economy in the East. Many countries around the world are very sceptical to import food products, which contain milk from China.

The only way to prevent future contaminations is to completely eradicate melamine from the feed production system. This can be achieved by a combined effort of all the parties involved, from the farmer to the producer, in the production of animal feeds and foods intended for human consumption.

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CHAPTER 3

THE TRANSMISSION OF MELAMINE FROM FERTILIZER TO PASTURE GRASS

Abstract

Melamine is a commercially available industrial chemical with a high nitrogen content. Large quantities of melamine waste can sometimes be incorporated into crop and pasture fertilizers due to the high N content. An initial pot plant trial with kikuyu was conducted to determine whether melamine would be absorbed as such from the soil to the plant material. The pots were fertilized with melamine adulterated Chinese maize Gluten 60. Results indicated that melamine was indeed absorbed and 7 days after fertilization, the concentration of melamine in the grass was 228 ppm. An applied pasture trial was then conducted where three adjacent pastures of 0.3 ha each were used. One pasture served as a control and received N fertilization in the form of LAN at a rate of 40 kg N/ha. The other two pastures received LAN with 10% (Treatment 1) and 20% (Treatment 2) of the N substituted by melamine, respectively. All three pastures also received P-fertilization in the form of Single Superphosphate at a rate of 20 kg P/ha and KCl at a rate of 50 kg K/ha. Pasture samples were taken once a week for 10 weeks, each time at the exact same spot in each camp. Samples were dried and finely milled before analysis via LC-MS/MS for melamine content. The melamine was present in the grass when harvested one week after fertilization. The rate at which melamine decayed in the plant material was found to be quite similar for the two melamine treatments. In this trial, melamine took around 10 weeks to reach undetectable levels in the grass. It was concluded that melamine was absorbed as such from the soil by pasture grass when included in a fertilizer.

3.1 Introduction

Kikuyu, or *Pennisetum clandestinum,* is one of the most popular pasture grasses for dairy cattle in South Africa. These planted pastures are highly palatable and, when managed correctly, they are digestible and have a low fibre and high crude protein content (van Oudtshoorn, 2006). Well managed kikuyu pastures can play a very important role in the production of milk in South Africa as many of the milking areas rely quite heavily on grazing systems for milk production.

One of the most important limitations to pasture growth is the low soil nitrogen (N) content in the tropics and sub-tropics (Marais, 2001). Nitrogen fertilized pastures have been shown to increase animal production and are more robust than non-fertilized pastures in their response to different management forms. Fertilization will increase dairy production as well as beef production. This is an economically important reason for the application of fertilizers. The increase in animal productivity will compensate for the high cost involved in the fertilization of pastures. Fertilization of pastures ensures that the fertility of the soil is high enough for pastures to grow at an optimum level and also to ensure that there are no imbalances in the soil caused by the loss of nutrients through leaching and/or grazing. Kikuyu reacts well to nitrogen fertilization and it can withstand heavy grazing (Botha, 2009).

Melamine is used in the production of many industrial and household products, but before melamine can be used to produce these products, it must first be produced in mass quantities. The production of melamine results in a large amount of melamine waste. The high nitrogen content of melamine (670 g/kg) would make the use thereof as a source of N for fertilization very attractive and the application as a crop fertilizer would sound like a feasible way to dispose of melamine waste. Most fertilizers are applied before or during the growing phase of crops or pastures. Melamine is said to have the slowest biodegradation rate of the triazine compounds (Hauck & Koshino, 1972) and studies have shown that a very small amount of nitrogen from melamine is converted to nitrate over a long period, which again confirms that melamine is a slow-release fertilizer component (Scholl *et al.*, 1937). The extremely slow release action of melamine would thus cause the nitrogen only to be available when the plants probably do not need it any more. Despite these poor characteristics, melamine is sometimes used as a fertilizer ingredient, which may be due to the surplus amounts available.

The aim of the study was to test the hypothesis that melamine, when included in a fertilizer, would be absorbed from the soil as such by the pasture grass, and to determine the rate of melamine depletion from the grass.

3.2 Materials and Methods

The study consisted of two parts, *viz*. a pilot pot plant trial and an applied pasture trial.

3.2.1 Location

The pot plant trial was conducted in a laboratory of the Department of Animal Sciences, Stellenbosch University, while the applied pasture trial was conducted at the Welgevallen Experimental Farm of the Stellenbosch University, Western Cape, South Africa (33° 55’ 12” S, 18° 51’ 36” E).

3.2.2 Treatments

Pilot pot plant trial

For the pot plant pilot trial, Kikuyu (*Pennisetum clandestinum*) was planted in four small 1.5 L plastic buckets with holes drilled into the bottom of the buckets to ensure adequate drainage of water. In two of the buckets, the grass was fertilized with melamine using maize Gluten 60 from Chinese origin that was contaminated with melamine. This Gluten 60 was the same that had been found in the feed of dairy cattle in some parts of the Western Cape and contained 15 117 mg/kg of melamine. The grass in the two buckets was fertilized at a rate equivalent to 8.8 kg melamine per hectare. The other two buckets served as control and did not receive any fertilization. Grass was watered regularly and the re-growth was cut weekly for melamine analysis.

Applied pasture trial

For the applied pasture trial, three adjacent Kikuyu (*Pennisetum clandestinum*) pastures of 0.3 ha were measured out and fenced. Before fertilization, all three pastures were mowed to a height of approximately 70 mm.

All three pastures were fertilized by hand with Single Superphosphate (8.3% P; Agricol Fertilizers, Eagle Str, Brackenfell, Western Cape, South Africa) at a rate of 20 kg P/ha and with KCl (50% K; Agricol Fertilizers, Eagle Str, Brackenfell, Western Cape, South Africa) at a rate of 50kg K/ha. All pastures received nitrogen at a rate of 40 kg/ha, but the form in which N was applied differed between pastures. The levels of N, P and K that were applied, were according to recommendations by Dr. Phillip Botha, pasture scientist of the Outeniqua Experimental Farm (2010, personal communication). Limestone ammonium nitrate (LAN; Agricol Fertilizers, Eagle Str, Brackenfell, Western Cape, South Africa) was used as the basis of the N-fertilization, but melamine replaced part of the LAN in two of the three treatments. The LAN contained 280 g/kg of N. The melamine that was used (99% pure) was supplied by Sigma-Aldrich, St. Louis, MO.

In Treatment 1, 10% of the LAN-N was replaced by melamine-N. The rate of application (on 0.3 ha) was thus 38.6 kg of LAN (equivalent to 129 kg/ha) and 1.79 kg of melamine (equivalent to 5.97 kg/ha melamine). In Treatment 2, 20% of the LAN-N was replaced by melamine-N. The rate of application (on 0.3 ha) in Treatment 2 was thus 34.3 kg of LAN (equivalent to 114 kg/ha) and 3.58 kg of melamine (equivalent to 11.94 kg/ha melamine). In Treatment 3, the pasture was fertilized with LAN as sole source of N at a rate of 47.7 kg (equivalent to 143 kg/ha). For Treatments 1 and 2, the LAN pellets and melamine powder were mixed in a cement mixer together with canola oil at a rate of 2 L (Treatment 1) and 4 L (Treatment 2) of oil per ton of fertilizer to prevent dustiness and to prevent the LAN pellets and melamine powder from separating.

Following fertilizer application, the pastures were irrigated at a rate of 25 mm immediately and at a rate of 25 mm twice a week thereafter. All pastures were allowed to grow for 28 days before the animals were allowed to graze them. This was to ensure that there was enough re-growth for the animals to graze. These three fertilized pastures were used in the milk production study as described in Chapter 4.

3.2.3 Sampling of Grass

Pilot pot plant trial

Grass in the plastic buckets was harvested by hand with scissors every 14 days for 4 months. With each cutting, the grass re-growth was cut to the same height, *viz*. 10 mm. After harvesting, grass was dried at 60˚C in a forced draught oven for approximately 2 days until constant mass. Samples were then finely milled with a Knifetec^{TM} 1095 mill (Foss, Hillerød, Denmark) and placed in identified plastic zip lock bags for storage until further analysis.

Applied pasture trial

After fertilization, pasture samples were taken once a week for melamine analysis, every time from exactly the same areas in the camps. For Treatments 1 and 2, samples were taken from nine spots, as schematically indicated in Figure 6. The sample areas were approximately 30 cm in diameter. To confirm that the pasture in Treatment 3 had no detectable levels of melamine, the control pasture was sampled at a single area on the same day each week.

The sampling areas of each pasture are illustrated in Figure 6. Pasture samples were cut with a hand held sheep shear down to where the leaves start to grow. For each treatment, samples A1 and A2 were pooled and placed into plastic bags. The same was done with B1 and B2, C1 and C2 and D1 and D2. Once in the laboratory, the pooled samples were cut into smaller pieces with a pair of scissors. The cut grass was then dried at 60˚C in a forced draught oven until constant weight was achieved after two days. Samples were then finely milled with a Knifetec™ 1095 mill (Foss, Hillerød, Denmark) and placed in identified plastic zip lock bags for storage until further analyses.

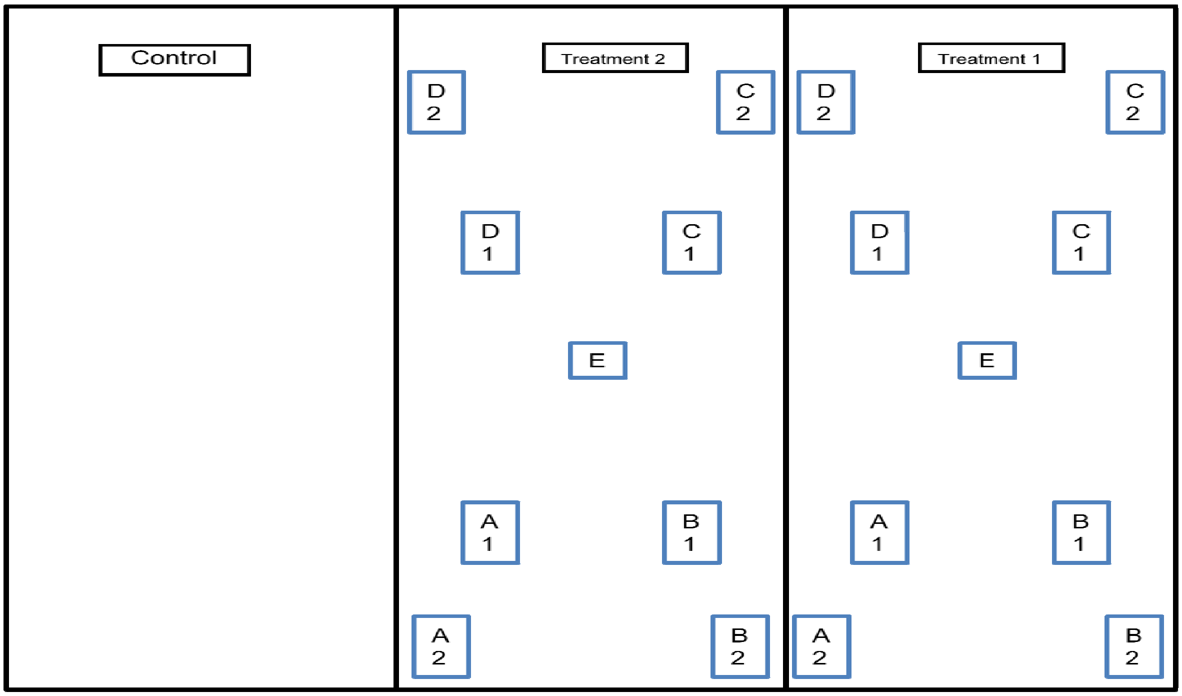

Figure 6 Sampling areas in the three different camps of the applied pasture trial.

3.2.4 Chemical analyses

Dry matter

Crucibles were accurately weighed on a four decimal point scale. One gram (g) of each sample was then weighed out into crucibles. Crucibles and samples were placed in a 100°C forced draught oven for 24 hours. After 24 hours, the crucibles were removed from the oven and placed in a desiccator for 30 minutes before their weight was once again accurately determined (AOAC, 2002; Method 934.041).

$$\%\ \text{Moisture} = \frac{\text{Sample weight (g)} \ - \ \text{Dry sample weight (g)}}{\text{Sample weight}} \times 100$$

$$\%\ \text{DM} = 100 - \%\ \text{Moisture}$$

Ash

Crucibles were weighed on a four decimal scale. One gram (g) of each sample was then accurately weighed into crucibles. The crucibles were placed in a 500°C muffle furnace for 6 hours. After the crucibles had cooled down enough to be removed from the muffle furnace, they were placed in a desiccator for 30 minutes to cool down and then weighed on a four decimal scale to determine the ash content of the samples (AOAC, 2002; Method 942.05).

$$\%\text{Ash} = \frac{(\text{Weight of crucible and ash}) - (\text{Weight of dry empty crucible})}{\text{Sample weight}} \times 100$$

$$\%\text{Organic matter} = 100 - \%\text{Ash}$$

Crude fibre

Crucibles were accurately weighed on a four decimal scale. One gram (g) of each sample was then weighed out into crucibles. Crucibles were placed in the extractor unit of a Fibertech apparatus (Fibertech System M, 1020 Hot extractor, SMM Instruments Pty. Ltd. Cape Town, South Africa). First, the valves of the Fibertech apparatus were closed followed by the opening of the water tap to cool the apparatus. The apparatus was checked for any leakages before the analyses commenced. After making sure no leakages were found, 150 mL of a boiling H_2SO_4 solution was added to each glass crucible and the temperature was set to 100°C. Once the solution boiled, the temperature was set to 65°C and the samples were gently boiled for another 30 minutes at 65°C.

After 30 minutes, the heat was turned off. The vacuum valves were opened and each sample was rinsed three times with distilled water. After rinsing, the valves were once again closed. The crucibles were removed from the Fibertech apparatus and placed in a 100°C forced draught oven for 24 hours. After 24 hours, the crucibles were removed and placed in a desiccator for 30 minutes to cool down before they were weighed on a four decimal scale. Immediately after weights had been recorded, the crucibles were placed in a 500°C muffle furnace for 6 hours. After removing crucibles from the furnace, they were placed in a desiccator for 45 minutes after which their weights were determined on a four decimal scale and recorded (AOAC, 2002; Method 962.09)

$$\%\text{Crude Fibre} = \frac{(\text{Residue in crucible after air drying}) - (\text{Residue in crucible after ashing})}{\text{Sample weight}} \times 100$$

Crude fat (Ether extract)

The aluminium fat beakers were placed in a 100°C forced draught oven for 24 hours to ensure they were moisture free. The beakers were placed in a desiccator for 30 minutes to cool down after they were removed from the oven. Beakers were then weighed on a four decimal scale and their weights were recorded. Next, 2 g of each sample was weighed out into the extraction thimbles and a piece of cotton wool was placed on top of each sample to prevent it from being flushed out of the thimbles. The beakers were then filled with 50 mL diethyl ether and the water flowing taps were opened for condensation. The apparatus used in determining the fat content of the samples was a Tecator Soxtec System HT 1043 Extraction Unit.

The oil bath, fan and heating were switched on. The thimbles were then transferred to the extraction tubes once the ready light flickered. Each individual thimble inside the extraction tubes matched the fat beaker on the element below the tubes. The extraction tubes were lowered onto the beakers with the handle of the extraction unit. Care was taken to ensure that the tubes were tightly sealed with each matching fat beaker. Next, the thimbles were lowered and boiled in the ether for 15 minutes (with the turned to “boiling” and the taps open). After 30 minutes, the thimbles were lifted out of the ether and the taps were closed for the ether to be collected, with the handle turned on “rinsing”. After the ether was collected, it was boiled for a further 15 minutes. The beakers were removed from the extractor unit and placed in a 100°C forced draught oven for 2 hours to evaporate all remaining ether from the beakers. After removing the beakers form the oven, they were placed in a desiccator for 30 minutes to cool down before they were weighed on a four decimal scale and the weights recorded.

When the analyses were completed, each beaker was cleaned out with 10 mL of diethyl ether, to remove the fat from the beakers and then washed out with distilled water. It can be noted that a small amount of H_2SO_4 can be used in addition to the diethyl ether if the fat residue remains in the beaker after cleaning. The H_2SO_4 should not be left in the beaker for more than 1 to 2 hours (AOAC, 2002; Method 920.39).

$$\%\mathrm{Fat} = \frac{(\text{Weight of Soxhlet beaker} + \text{Fat}) - (\text{Weight of Soxhlet beaker})}{\text{Sample weight}} \times 100$$

Nitrogen (Crude protein)

A Leco Nitrogen Gas Analyzer FP528 (LECO Africa (Pty) Ltd, Kempton Park, South Africa) was used to determine the total nitrogen content of the samples. Before the analysis commenced, the Leco apparatus was standardized in accordance with the manufacturer's instructions. An empty aluminium foil cup was placed on a four decimal scale and the scale was then zeroed. Next, ~0.1 g of the sample was accurately weighed into the cup and the weight recorded. The cup was then closed (by twisting the ends together) and was once again weighed and the weight recorded. The closed cup was placed onto the carousel sample tray and the Leco was switched on, with the temperature set to 850°C. After the samples had been combusted inside the Leco's furnace, the nitrogen content was recorded in percentage (%). To determine the crude protein of each sample, the nitrogen content was multiplied by 6.25 (AOAC, 2002).

Melamine analysis

The grass samples of both the pilot pot plant trial and the applied pasture trial were analyzed for melamine content with the same adapted method of Shai *et al.* (2008). The milled samples were prepared by extracting 0.20 – 0.30 g quantities with 5 ml of a solution consisting of acetonitrile (50%) and 0.1% formic acid, followed by sonication for 1 hour. Samples were then filtered and sample vials were loaded with 900 µL of the sample together with 100 µL of a 0.5 mg/kg stable isotope-labelled melamine ($^{13}C_3H_6{}^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). Samples were analysed by liquid chromatography tandem mass spectrometry

(LC-MS/MS) with a Waters API Quattro Micro triple quadruple mass spectrometer, connected to a Waters 2690 HPLC (Waters Corp., Milford, MA). This method has a 0.001 mg/kg detection limit of melamine for grass samples.

3.3 The estimation of kinetic coefficients

The solver option in Microsoft Office Excel and the following one component exponential model were used to calculate the kinetic coefficients from the melamine grass data and to construct the decay curve (Wylie *et al.,* 2000):

$$Y = a(e^{-kt})$$

Y = melamine concentration at time t

a = maximum melamine concentration

k = rate at which a decreased over time

t = time (weeks)

3.4 Results and Discussion

3.4.1 Pilot pot plant trial

Melamine concentration in the grass and the decay over time is presented in Figure 7.

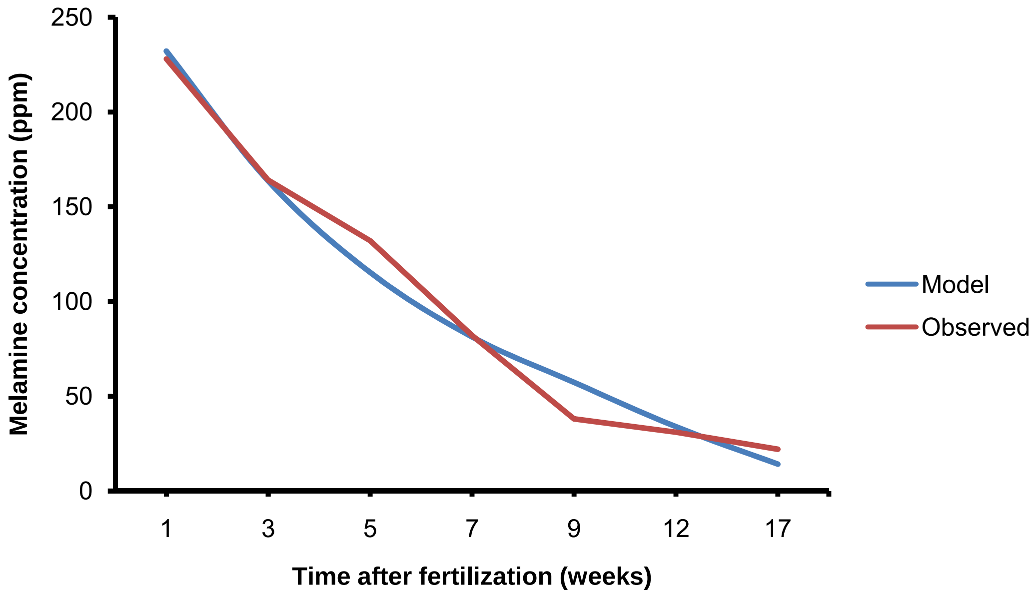

Figure 7 The effect of melamine application to kikuyu grass on the melamine content. Grass was planted in 1.5 L buckets and melamine was applied at an equivalent rate of 8.8 kg/ha via tainted maize Gluten 60 that contained 15 117 mg/kg of melamine.

Melamine was observed in the grass one week after fertilization already, which clearly demonstrates that melamine is absorbed from the soil as such. Seven days after fertilization, 288 mg/kg (ppm) of melamine was found in the harvested grass. The ratio between melamine concentration in the grass and that in the fertilizer was 0.015 (or 1.5%). From Figure 7 it is evident that melamine depletion from the vegetative parts of the grass followed a non-linear pattern. Melamine concentration decreased at a rate of k = 0.17 so that the concentration at 119 days (17 weeks) in the experimental plant material was 22 mg/kg.

Most countries, including South Africa, have accepted the guidelines set by the FDA (CNN, 2008) that foods for human consumption should contain less than 2.5 mg/kg of melamine (for infant formula the maximum level is 1 mg/kg). Feed companies have also adopted the 2.5 mg/kg guideline for animal feeds (AFMA, 2009). Based on the level of fertilization applied in the pot plant trial, it would take 27 weeks for the grass to reach levels below 2.5 mg/kg.

3.4.2 Applied pasture trial

Results of the applied pasture trial are presented in Figure 8. The pasture melamine data were fitted to the decay curve $Y=a(e^{-kt})$ where “a” represents the maximum melamine concentration and “k” represents the rate at which “a” can be expected to decrease over time.

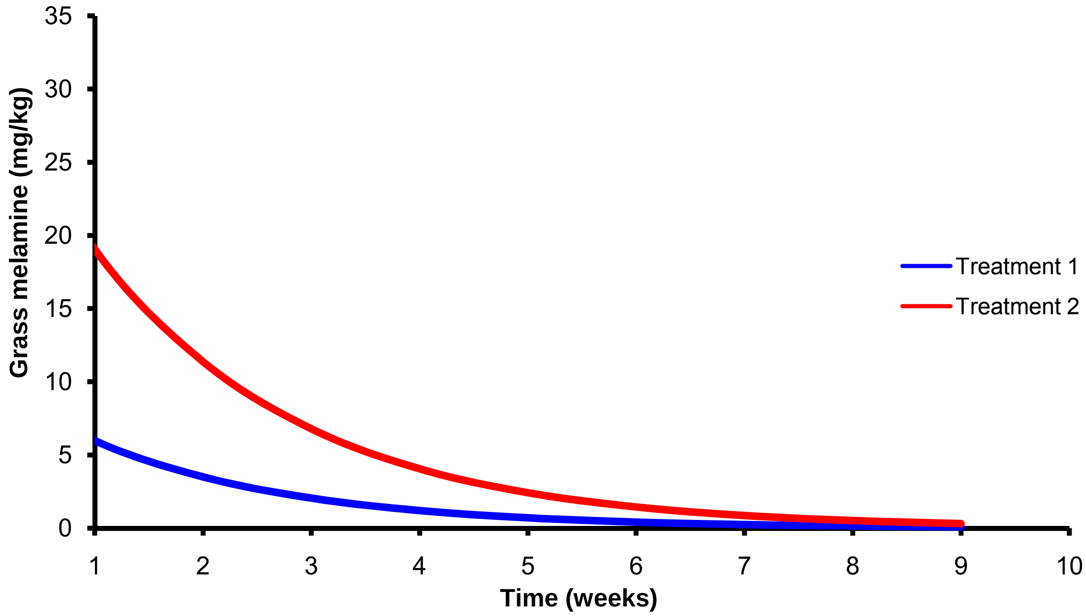

Figure 8 The effect of fertilization of kikuyu pastures on melamine concentration. Treatment 1: Melamine-N replaced 10% of the LAN-N. Treatment 2: Melamine-N replaced 20% of the LAN-N.

For each treatment, the melamine concentrations presented in Figure 8 are the mean values of samples taken on 9 different spots on the pastures. As expected, the a-values of the two melamine fertilized camps differed significantly from each other. It appeared that the higher melamine application (20% of LAN-N replaced by melamine-N) resulted in a three-fold higher pasture melamine concentration one week after fertilization than the lower application (10% of LAN-N replaced by melamine-N). The efficiency of melamine absorption from the soil may therefore be a function of application rate.

The k-values, which represent the rate at which melamine would decrease in the pasture over time, were quite similar. For Treatment 1, k = 0.54 and for Treatment 2, k = 0.52. This would suggest that, despite the initial concentration of melamine in the fertilizer, the rate at which melamine decreases in pasture would almost be the same. In this study, it took more than 10 weeks for the melamine to reach undetectable levels. It is well known that melamine has been used as a slow release N source for many years. However, the fact that melamine is also absorbed by pastures makes it a potentially dangerous N-fertilizer.

The results of the chemical analysis of the grass during week 4, which was the time that the cows were put onto the pastures, are presented in Table 2. Substituting a portion (10% and 20%) of the fertilizer LAN with melamine did not seem to have an effect on the chemical composition of the grass. The nitrogen and crude protein levels did not differ much, which can be attributed to the fact that the same amount of N was applied to all three pastures, whether it was in the form of LAN, or melamine substituted fertilizer. The crude fibre of Treatment 2 was higher than that of the other two pastures. It was also observed during the trial period that the grass from Treatment 2 looked less lush and green than that of Treatment 1 and Control.

Table 2 Chemical composition (g/kg) of grass during week 4 of the applied pasture trial. All values are on DM basis.

	Control	Treatment 1	Treatment 2	Average ± SE	CV
DM	959.20	926.36	930.50	938.96 ± 10.33	1.91
Ash	97.20	97.88	80.46	91.85 ± 5.70	10.74
Crude fat	37.60	29.84	29.14	32.19 ± 2.71	14.58
Crude fibre	207.10	283.36	296.50	262.32 ± 27.87	18.40
Nitrogen	30.30	32.74	28.62	30.55 ± 1.20	6.78
Crude protein	189.38	204.63	178.88	190.96 ± 7.84	6.78

DM = Dry matter

SE = Standard Error;

CV = Coefficient of Variation

3.5 Conclusion

In this study, it was found that melamine in fertilizers can be absorbed as such from the soil by the pasture grass. The mechanism of absorption is not known. After a single application of fertilizer, the melamine concentration in the pasture can be expected to decrease over time. In this trial, grass was sampled for melamine analysis from the same locations on the pasture every week. The pasture regrowth was thus analysed. The melamine depletion rate in uncut pasture grass is unknown. Therefore, when pasture contamination is suspected, it would be advisable to take samples for melamine analysis on a weekly basis and continue to do so until melamine reaches undetectable levels. Stricter policies should also be put in place for fertilizer manufacturers as to prevent the use of melamine and its by-products in the manufacturing of fertilizers for crops and pastures. This would ensure that melamine stays out of the food chain.

Future research should investigate the effect of melamine on the growth rate and production potential of various plant species, and secondly the effect on humans and animals when melamine enters the food and feed chains via fertilisers.

3.6 References

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CHAPTER 4

THE TRANSMISSION OF MELAMINE FROM PASTURE TO MILK

Abstract

Eighteen lactating Holstein cows, 60 ± 5.1 (SE) DIM, with a daily milk production of 36.5 ± 2.0 (SE) kg/d and weighing 609 ± 12.8 (SE) kg, were stratified according to milk production and then randomly allocated to 3 groups of 6 cows. The groups were then randomly allocated to the three pastures used in the applied pasture trial. In the two melamine treatments, melamine was applied to kikuyu pastures via LAN fertilizer at rates of 6.0 kg/ha (Treatment 1) and 12.0 kg/ha (Treatment 2). Cows were kept on the melamine fertilized pasture for 9 days, in which they were allowed to graze the pasture for approximately 10 hours each day. After the 9 day period, melamine was withdrawn by placing the cows on the control pasture that did not receive melamine fertilization for another 7 days. During these 15 days, milk was collected twice a day, viz. during the morning and afternoon milkings. Milk from the cows placed on melamine fertilized pasture was discarded to prevent contamination of the other milk of the rest of the herd. The milk was then pooled and sub-divided into two samples, one was preserved with potassium dichromate and analysed for milk composition and the other was frozen and analysed for melamine by LC-MS/MS. Results from the analysis for melamine confirmed that melamine is transferred from melamine fertilized pasture to milk. The mean transfer efficiency of melamine to milk was 3.0% in Treatment 1 and 2.1% in Treatment 2. In this study, it took 6 days from melamine withdrawal for melamine to reach undetectable levels in the milk. It was also found that the melamine fertilized pasture did not have any significant effect on the average milk production and milk composition of the cows. The aim of the study was met and it was confirmed that melamine will be transferred from melamine fertilized pasture to the milk of cows grazing these pastures.

4.1 Introduction

The presence of melamine in milk is not new to society. Worldwide there are many cases of melamine-tainted milk. China is very well known for their role in a melamine scandal in 2008. Around 296 000 babies had fallen ill and 6 died due to illnesses related to the consumption of melamine tainted infant formula (All about feed, 2009). Melamine can make its way into milk via various routes. The scandal in China was caused by melamine being added to watered down milk to increase the apparent protein content. Xiu and Klein (2010) stated in their review of the factors that lead to the deliberate use of melamine, that melamine was being sold readily in villages. A white "protein powder" was available, with instructions on how to mix it with watered down milk. It should be kept in mind that many of the dairy farmers in China are poor and uneducated and milk buyers paid more money for the higher protein milk. This might have been one of many reasons why melamine was deliberately added to milk. Melamine can also find its way into milk purely by accident. Gluten imported from China by South Africa, had been found to contain high levels of melamine and cyanuric acid, among other things. South African feed manufacturers used this tainted gluten in feeds and concentrates, oblivious of the fact that it contained melamine, and animals ingested the tainted feed. Cruywagen et al. (2009) did a trial to determine if melamine could be carried over from tainted feed to milk. Results from this trial confirmed that melamine can in fact be carried over from the feed to the milk.

Melamine is sometimes used as a crop or pasture fertilizer in the agricultural industry. This is due to the fact that melamine has a high nitrogen content (670g/kg nitrogen on a molecular basis; Merck, 1987). Melamine is a slow-release nitrogen fertilizer, but the fact that it is readily available and much less expensive than other nitrogen sources, makes it an attractive ingredient in fertilizers.

In the current study, cows were placed on the pastures that were discussed in the previous chapter, to test the hypothesis that melamine would be transferred to milk when cows graze pasture that had been fertilized with melamine containing fertilizers. A further objective of the study was to determine the rate and efficiency of melamine transmission from pasture to milk.

4.2 Materials and Methods

4.2.1 Location

The milk production study was conducted at the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa (33° 55’ 12” S, 18° 51’ 36” E). The trial protocol was approved by the Stellenbosch University’s Animal Ethics Committee (Reference: ACU/2010/009).

4.2.2 Animals

Eighteen lactating Holstein cows, 60 ± 5.1 (SE) DIM, with a daily milk production of 36.5 ± 2.0 (SE) kg/d and weighing 609 ± 12.8 (SE) kg, were stratified according to milk production and then randomly allocated to 3 groups of 6 cows.

All the cows in the trial were fed a commercial semi-complete pelleted diet at a rate of 22 kg/cow/day. Feeding took place twice a day, at 07h00 and 16h00, respectively. Treatments and grazing schedule is explained below.

4.2.3 Treatments

The three groups of cows all received different treatments, based on fertilizer application to the pastures. The groups were randomly allocated to the three pasture camps. The treatments and fertilizer applications were as discussed in Chapter 3.3.3. Briefly, all pastures received N at a rate of 40 kg/ha. Limestone ammonium nitrate (LAN) was used as the basis of N-fertilization, but melamine replaced part of the LAN in two of the three treatments. In Treatment 1, 10% of the LAN-N was replaced by melamine-N and in Treatment 2, the substitution was 20%. In Treatment 3 (Control), the pasture was fertilized with LAN as sole source of N. Pastures were allowed to grow for 28 days before allowing cows to graze. This is was to ensure that enough DM was available for the cows to graze. Cows were turned out to pasture every morning at 07h00 after milking. From 11h00 to 14h30, they were kept together in a small free-stall barn where they received a semi-complete commercial pelleted feed. At 16h30, after the afternoon milking, cows were returned out to pasture again until the following morning. Actual grazing time was estimated to be approximately 10 hours per day. Cows in Treatments 1 and 2 were placed on the melamine fertilized pastures for 9 days, after which all the cows were switched to the control pasture for another 7 days.

4.2.4 Collection of milk samples

Milk samples were collected daily from each cow during the morning and afternoon milkings. For the duration of the trial, milk production was recorded individually at each milking. Milk was collected in 250 mL plastic bottles with screw lids, which had been labelled with each cow's number and then taken to the laboratory for further sub-sampling. Two sub-samples of approximately 45 mL were taken for each cow and were either frozen at −18° C or preserved with potassium dichromate. The frozen samples were later analyzed for melamine, while the preserved samples were analyzed for milk composition.

During the first three days, the milk of both the morning and afternoon milkings was sub-sampled. From the fourth day, milk was pooled proportionally for each cow according to the morning and afternoon milk yields. In other words, for each litre of milk produced, 10 mL of the sample was taken.

For the duration of the trial, and until no melamine could be detected in the milk any more, milk from the melamine treated cows were harvested separately and after all the other cows had been milked. The melamine contaminated milk was discarded by in the manner which was approved by the Stellenbosch University's Animal Ethics Committee (Reference: ACU/2010/009).
Milk from cows in the melamine treatments could thus not contaminate milk collected from the rest of the herd.

4.2.5 Analysis of milk samples

Milk samples which had been preserved with the potassium dichromate, were analysed in the Dairy Laboratory of the ARC-Elsenburg Analytical Services for milk protein, milk fat, lactose, somatic cell count, milk urea nitrogen (MUN) and total solids by means of a MilkoScan FT 6000 (Foss, Hillerød, Denmark).

An adapted method of Shai *et al.* (2008) was used to analyse the frozen milk samples for melamine. Frozen samples were thawed and then diluted with 0.2 *M* perchloric acid on a 1:1 basis. The diluted samples were then placed in a centrifuge for 5 minutes at 5000 rpm. Strong cation-exchange solid-phase extraction cartridges (Phenomenex Strata SCX, 55 µm, 70 A, 500 mg/3 mL, provided by Separations, Randburg, South Africa) were treated with 6 mL of methanol followed by 6 mL of water. Cartridges were loaded with 3 mL of the milk supernatant and 100 µL of a 0.5 ppm stable isotope-labelled melamine ($^{13}C_3H_6{}^{15}N_3$) internal standard solution (Cambridge Isotope Laboratories Inc., Andover, MA). The supernatant was allowed to slowly pass through the cartridge. Cartridges were washed with 6 mL of 0.1 *N* HCl followed with 6 mL of methanol and then aspirated for 1 minute under a vacuum. Melamine was then washed out into a clean tube with 6 mL of ammonium hydroxide:methanol:dichloromethane (1:5:5). Extracts were dried by placing them under a stream of nitrogen before they were resuspended in 1 mL of acetonitrile (50%). Samples were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) with a Waters API Quattro Micro triple quadruple mass spectrometer, connected to a Waters 2690 HPLC (Waters Corp., Milford, MA). This method has a 0.005 mg/kg detection limit for milk samples.

4.3 Results and Discussion

The results of the milk production study are presented in Table 3.

Table 3 The effect of melamine as fertilizer ingredient on milk production and milk composition of cows.

Item	Low melamine[1]	High melamine[2]	Control	SEm	P
Milk production (kg/d)	29.0	28.1	29.8	2.43	0.892
Milk fat (g/kg)	32.5	30.8	32.9	2.16	0.762
Milk protein (g/kg)	28.4	31.0	29.9	0.98	0.279
Lactose (g/kg)	46.1	47.2	46.7	0.81	0.621
Total solids (g/kg)	116	116	117	0.28	0.973
Milk urea N (mg/dL)	13.7	12.9	13.8	0.35	0.193

[1]Low melamine = 10% of the N in LAN fertilizer was replaced by melamine-N.

[2]High melamine = 20% of the N in LAN fertilizer was replaced by melamine-N.

SEm = Standard error of the mean.

P = Significance level.

From Table 3 it can be seen that the melamine as fertilizer ingredient had no effect on milk composition. All values were within the normal ranges and the values observed in the cows receiving melamine fertilized pasture did not differ from the values of the control group. This is in agreement with Cruywagen *et al.* (2009), Battaglia *et al.* (2010) and Shen *et al.* (2010), who all reported no effects of dietary melamine in milk production or milk composition.

Results of the effect of melamine fertilized pastures in milk production and milk melamine concentration are presented in Figure 9.

The first appearance of melamine in the milk was in the afternoon milk of the first day that the cow grazed the melamine fertilized pastures, which was approximately 8 hours after first ingestion of melamine. The observed melamine concentrations were 20.6 μg/kg for Treatment 1 and 13.0 μg/kg for Treatment 2. It is not clear why the melamine concentration in the milk was higher in Treatment 1 than in Treatment 2 so soon after first ingestion. In Treatment 1, melamine concentrations did not get any higher, but in Treatment 2, melamine concentrations in the milk increased rapidly on day 2 (37 µg/kg) and reached a maximum value of 42 µg/kg on day 3. For the duration of the melamine ingestion period, milk melamine concentrations were, on average, about 2.5 times higher for Treatment 2 than for Treatment 1.

An interesting observation from Figure 9 is the sudden drop in the concentration of melamine in the milk on day 5 and day 8. One can speculate that this observation might be because the pastures have been fertilized by hand (as indicated in Chapter 3, section 3.2.2) and even though the fertilizer had been applied as evenly as possible, some areas in the pasture might have received more fertilizer that others, giving it a higher melamine concentration. As illustrated in Figure 6, pasture grass was sampled from nine areas in the two melamine treated camps to compensate for the possibility that a single sampling area might have received less fertilizer than another area, thus influencing the values for predicted melamine intake. Results from the melamine analysis of each sampling area varied greatly from each other and an average melamine concentration per day in the pasture grass was used for each pasture. Due to the variable nature of cows' grazing patterns, it can be hypothesized that, on days 5 and 8, the cows grazed areas in the pastures that received less fertilizer. However, the phenomenon was observed on the same days for both Treatments 1 and 2. It is therefore highly unlikely that possible variations in pasture melamine concentrations were responsible for the lower milk melamine concentrations observed on these days. A more acceptable explanation would be that the pasture intake was much lower on these days compared to the rest of the trial period. There were a few hot days during the trial period and it might have reduced pasture intake on these days. Unfortunately, daily temperatures were not recorded during the trial.

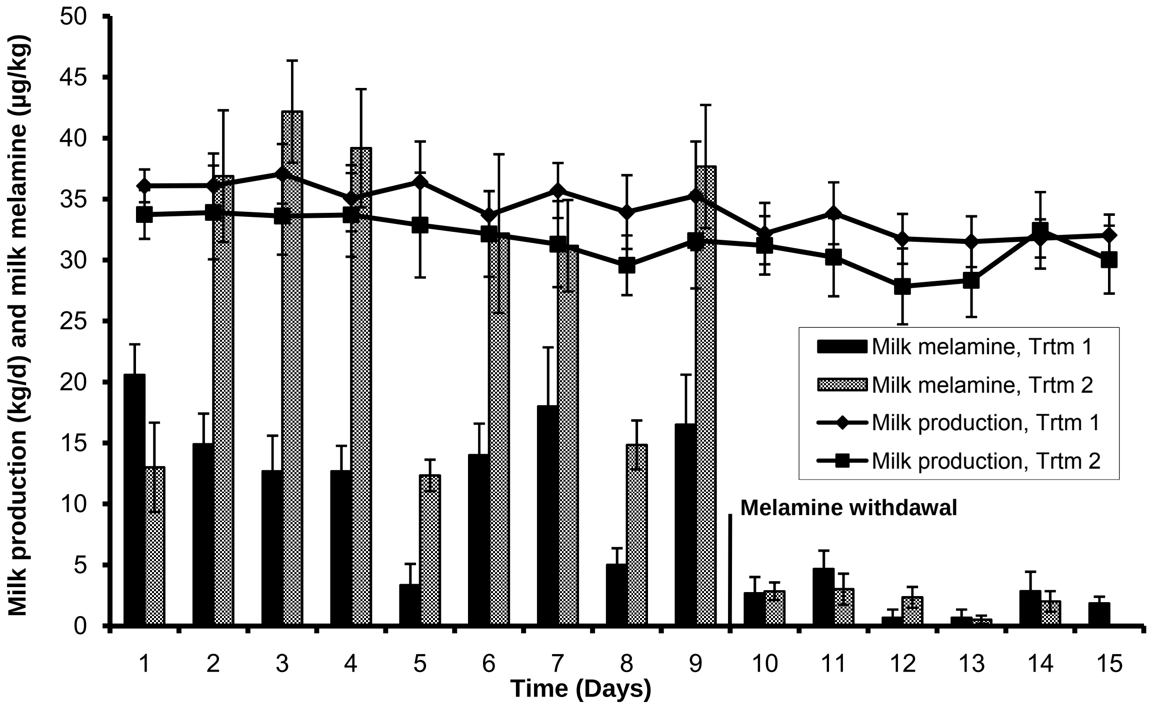

Figure 9 Milk production and melamine concentration.

The efficiency of melamine transfer from pasture to milk was calculated as the ratio between melamine ingestion (mg/day) and melamine excretion via milk (mg/day), expressed as a percentage. During the eight days that the cows grazed the melamine fertilized pasture, the mean transfer efficiency was 3.02% for Treatment 1 and 2.10% for Treatment 2. These values agree with the observation of Cruywagen *et al.* (2009) who reported a transfer efficiency of 2.1% and Battaglia *et al.* (2010) who reported transfer efficiencies between 2.3 and 3.3%. Efficiencies were, however, much higher than those reported by Shen *et al.* (2010) *viz.* 0.66 to 0.95%. After withdrawal from contaminated pastures, it took 6 days for milk melamine to reach undetectable levels.

Most countries have accepted the guidelines set by the FDA (CNN, 2008) that foods for human consumption should contain less than 2.5 mg/kg of melamine (for infant formula the maximum level is 1 mg/kg). Feed companies have also adopted the 2.5 mg/kg guideline for animal feeds (AFMA, 2009). Results from the current study indicate that when melamine was applied to a pasture as fertilizer ingredient at rates of 5.97 kg/ha and 11.94 kg/ha (as indicated in Chapter 3, section 3.2.2), melamine appeared in the milk, but it never reached levels near the maximum allowed for human foods. On average, milk contains approximately 12% solids. Therefore, when milk is evaporated to manufacture milk powder, which is the main ingredient of dairy based infant formulae, the solids content would increase 8.3 fold. In the current study, the highest melamine concentration observed was 42 ug/kg. Upon drying, the concentration would thus increase to approximately 350 ug/kg, which would be approximately 7 times lower than the maximum allowable level of 2500 ug/kg for human consumption and almost 3 times lower than the 1000 ug/kg limit set for infant formula. Therefore, melamine transfer form fertilizer to pasture still remains a potential danger. It should also be kept in mind that the transfer efficiency of melamine from fertilized pasture to milk could be affected by various environmental factors, such as fertilizer application rate, soil mineral status, rate of pasture growth, irrigation, rainfall and temperature. In the current study, Kikuyu was almost at the end of its productive growth curve. In the spring and early summer when pastures grow more actively, melamine might even be absorbed at a higher rate.

4.4 Conclusion

The objective of this study was met and it was found that melamine is in fact transferred to the milk of cows that graze melamine fertilized pasture. Melamine occurred in the milk as soon as 8 hours after cows grazed the melamine tainted pasture. The efficiency of melamine transfer from grass to milk was 3% for the low melamine concentration pasture and 2.1% for the high melamine concentration pasture. There are still a number of ways in which melamine can find its way into milk and indirectly into the food chain. Some of these ways are not necessarily a result of adulterations with melamine. They are simply common agricultural practice, such as the fertilization of pasture grass. As mentioned in the previous chapter, a lot of work is still needed to completely eradicate melamine from animal feeds and the food chain. One of the ways in which this can be done is to regulate the ingredients of agricultural fertilizers. This would help to prevent the use of melamine as a fertilizer ingredient in the future.

4.5 References

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CHAPTER 5
GENERAL CONCLUSION

In the pot plant trial, which was a pilot study to determine if melamine applied as fertilizer would be transferred from soil to grass, it was found that melamine appeared at a concentration of 288 mg/kg (ppm) in the grass seven days after fertilization. Based on the guidelines set by the FDA that 2.5 mg/kg melamine is allowed in animal feed, it was calculated that it would take 27 weeks for the fertilized grass to reach levels below 2.5 mg/kg. This is quite a long time when considering the fact that the grass received fertilization at a rate equivalent to 8.8 kg melamine per hectare.

In the applied pasture trial, it was found that melamine, when it replaced 10 or 20% of the N in a limestone ammonium nitrate fertilizer, was absorbed from the soil by the pasture grass. The melamine concentration decreased in the pasture over time. The exact time taken for melamine to reach undetectable levels would depend on the application rate, season and climatic conditions. Such information would be important as to assist one in determining the time in which animals should be kept off pastures, which had been found to be fertilized with products containing melamine or any of the melamine by-products. This should minimise the risk in contaminating milk with melamine *via* the pasture pathway. Pastures should be tested for traces of melamine to avoid accidental contamination of milk *via* the pastures, which have been fertilized with melamine containing fertilizer.

The objective of the milk production study was met and it was found that the melamine was in fact transferred from fertilizer to the soil, to the grass, and eventually to the milk of the cows that grazed the contaminated pasture. Melamine occurred in the milk as soon as 8 hours after cows had grazed the melamine tainted pasture. After withdrawal from contaminated pastures, it took 6 days for milk melamine to reach undetectable levels. The efficiency of melamine transfer from grass to milk was 3% for the low melamine concentration pasture and 2.1% for the high melamine concentration pasture. There are still a number of ways in which melamine could find its way into milk and indirectly into the food chain. Some of these ways are not necessarily a result of adulterations with melamine. They are simply common agricultural practice, such as the fertilization of pasture grass.

A lot of work is still needed to completely eradicate melamine from animal feeds and the food chain. One of the ways in which this can be done, is to regulate the ingredients of agricultural fertilizers. This would help to prevent the use of melamine as a fertilizer ingredient in the future.

Stricter policies should also be put in place for fertilizer manufacturers as to prevent the use of melamine and its by-products in the manufacturing of crop and pasture fertilizers. This would ensure that melamine stays out milk and in turn, out of the food chain.